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Bacillus Diphtheriae Real Time PCR Kit User Manual
For In Vitro Diagnostic Use Only

REF **RD-0062-02**

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For use with ABI Prism®7000/7300/7500/7900/Step One Plus; iCycler iQ™4/iQ™5; Smart Cycler II;Bio-Rad CFX 96;Rotor Gene™6000; Mx3000P/3005P;MJ-Option2/Chromo4; LightCycler®480 Instrument

EC REP Obelis S.A.
Boulevard Général Wahis 53
1030 Brussels, BELGIUM
Tel: +(32) 2.732.59.54
Fax: +(32) 2.732.60.03
E-Mail : mail@obelis.net

Shanghai ZJ Bio-Tech Co., Ltd.
www.liferiver.com.cn Tel: +86-21-34680596
trade@liferiver.com.cn Fax: +86-21-34680595
2nd floor,No.15 Building,No.188 Xinjunhuan road,
PuJiang Hi-tech Park Shanghai China

1. Intended Use

Bacillus Diphtheriae real time PCR Kit is used for the detection of Bacillus Diphtheriae by real time PCR systems in samples like nasal and pharyngeal secretions, sputum, C.S.F. and etc.

2. Principle of Real-Time PCR

The principle of the real-time detection is based on the fluorogenic 5'nuclease assay. During the PCR reaction, the DNA polymerase cleaves the probe at the 5' end and separates the reporter dye from the quencher dye only when the probe hybridizes to the target DNA. This cleavage results in the fluorescent signal generated by the cleaved reporter dye, which is monitored real-time by the PCR detection system. The PCR cycle at which an increase in the fluorescence signal is detected initially (Ct) is proportional to the amount of the specific PCR product. Monitoring the fluorescence intensities during Real Time allows the detection of the accumulating product without having to re-open the reaction tube after the amplification.

3. Product Description

Bacillus Diphtheriae, also known as Bacillus diphtheriae and Klebs-Löffler bacillus, is a pathogenic bacterium that causes diphtheria. C. diphtheriae is a facultatively aerobic Gram positive organism, characterized by non-encapsulated, non-sporulated, immobile, straight or curved rods with a length of 1 to 8 µm and width of 0.3 to 0.8 µm, which form ramified aggregations in culture (looking like "Chinese characters"). The bacterium may contain polymetaphosphate aggregates called Volutin granules. It is pathogenic only in humans. Many strains of C. diphtheriae produce diphtheria toxin, a proteic exotoxin which inhibit protein synthesis and thus is responsible for the signs of diphtheria. However, not all strains are toxigenic; the ability to produce the exotoxin is conferred on the bacterium when it is infected by a bacteriophage. Bacillus Diphtherae real time PCR kit contains a specific ready-to-use system for the detection of Bacillus Diphtherae by polymerase chain reaction (PCR) in the real-time PCR system. The master contains reagents and enzymes for the specific amplification of the Bacillus Diphtherae DNA. Fluorescence is emitted and measured by the real time systems' optical unit during PCR. The detection of amplified Bacillus Diphtherae DNA fragment is performed in fluorimeter **channel FAM** with the fluorescent quencher BHQ1. DNA extraction buffer is available in the kit. An external positive control(1×10^7copies/ml) contained, allow the determination of the gene load. For further information, please refer to section 9.2 Quantitation.

4. Kit Contents

Ref.	Type of Reagent	Presentation 25rxns
1	DNA Extraction Buffer	2 vials, 1.5ml
2	B. Diphtheriae Reaction Mix	1 vial, 950µl
3	PCR Enzyme Mix	1 vial, 12µl
4	Molecular Grade Water	1 vial, 400µl
5	B. Diphtheriae Positive Control(1×10^7copies/ml)	1 vial, 30µl

Analysis sensitivity: 1×10^3 copies/ml; LOQ: 2×10^3～1×10^8 copies/ml

Note: Analysis sensitivity depends on the sample volume, elution volume, nucleic acid extraction methods and other factors .If you use the DNA extraction buffer in the kit, the analysis sensitivity is the same as it declares. However, when the sample volume is dozens or even hundreds of times greater than elution volume by some concentrating method, it can be much higher.

5. Storage

- All reagents should be stored at -20°C. Storage at +4°C is not recommended.
- All reagents can be used until the expiration date indicated on the kit label.
- Repeated thawing and freezing (> 3x) should be avoided, as this may reduce the sensitivity of the assay.
- Cool all reagents during the working steps.
- Reaction mix should be stored in the dark.

6. Additionally Required Materials and Devices

- Biological cabinet
- Real time PCR system
- Trypsin digestive Solution
- Vortex mixer
- Real time PCR reaction tubes/plates
- Cryo-container
- Pipets (0.5 µl – 1000 µl)
- Sterile filter tips for micro pipets
- Sterile microtubes
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator and Freezer
- Desktop microcentrifuge for "eppendorf" type tubes (RCF max. 16,000 x g)
- Tube racks

7. ⚠Warnings and Precaution

Carefully read this instruction before starting the procedure.

- For in vitro diagnostic use only.
- This assay needs to be carried out by skilled personnel.
- Clinical samples should be regarded as potentially infectious materials and should be prepared in a laminar flow hood.
- This assay needs to be run according to Good Laboratory Practice.
- Do not use the kit after its expiration date.
- Avoid repeated thawing and freezing of the reagents, this may reduce the sensitivity of the test.
- Once the reagents have been thawed, vortex and centrifuge briefly the tubes before use.
- Prepare quickly the Reaction mix on ice or in the cooling block.
- Set up two separate working areas: 1) Isolation of the RNA/ DNA and 2) Amplification/ detection of amplification products.
- Pipets, vials and other working materials should not circulate among working units.
- Use always sterile pipette tips with filters.
- Wear separate coats and gloves in each area.
- Avoid aerosols

8. Sample Collection, Storage and transport

- Collect samples in sterile tubes;
- Specimens can be extracted immediately or frozen at -20°C to -80°C.
- Transportation of clinical specimens must comply with local regulations for the transport of etiologic agents

9. Procedure

9.1 DNA-Extraction

DNA extraction buffer is contained in the kit. Please thaw the buffer thoroughly and spin down briefly in the centrifuge before use. It's better to use commercial kits for nucleic acid extraction.

9.1.1 Sputum samples

1) **Trypsin digestive Solution preparation**

Add 10g trypsin to 200ml purified water and mix thoroughly. Adjust PH value to 8.0 with 2%NaOH solution. Add 2mL CaCl2 (25mmol/L), mix thoroughly and store at 4℃. **Please incubate at 37℃ for 10 minutes before use.**

2) Estimate the volume of the sputum and add partes aequales of the Trypsin digestive Solution then vortex vigorously. Set at room temperature for 30 minutes. Transfer 0.5ml mixture to a new tube. Centrifuge the tube at 13000rpm for 5 minutes, carefully remove and discard supernatant from the tube without disturbing the pellet.

3) Add 1.0ml normal saline. Resuspend the pellet with vortex vigorously**.** Centrifuge at 13000rpm for 5 minutes. Carefully remove and discard supernatant from the tube without disturbing the pellet.

4) Repeat step 3)

5) Add 100µl DNA extraction buffer, close the tube then suspend the pellet with vortex vigorously**.** Spin down briefly in a table centrifuge.

6) Incubation the tube for 10 minutes at 100°C.

7) Centrifuge the tube at 13000rpm for 10 minutes. The supernatant contains DNA extracted and is used for PCR template.

9.1.2 Nasal and pharyngeal secretions or C.S.F. samples

1) Take 1ml sample in a tube, centrifuge the tube at 13000rpm for 2min, and remove the supernatant and keep the pellet.

2) Add 100µl DNA extraction buffer to the pellet, close the tube then vortex for 10 seconds. Spin down briefly in a table centrifuge.

3) Incubate the tube for 10 minutes at 100°C.

4) Centrifuge the tube at 13000rpm for 10 minutes. The supernatant contains the DNA extracted and can be used for PCR template.

Attention:

A. During the incubation, make sure the tube is not open, as the vapor will volatilize into the air and may cause contamination in case the sample is positive.

B. The extraction sample should used in 3 hours or store at -20°C for one month.

C. DNA extraction kits are available from various manufacturers. You can also use your own extraction systems or the commercial kit depending on the yield. For DNA extraction, please comply with the manufacturer's instructions.

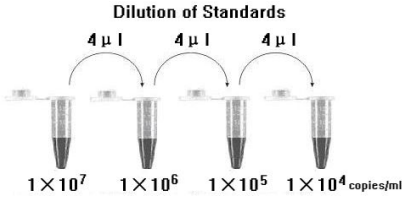
9.2 Quantitation

The kit can be used for **quantitative** or **qualitative** real-time RT-PCR. A positive control defined as 1×10^7copies/ml is supplied in the kit.

For performance of quantitative real-time PCR, Standard dilutions must prepare first as follows. Molecular Grade Water is used for dilution.

Dilution is not needed for qualitative real-time PCR detection.

Take positive control (1×10^7copies/ml) as the starting high standard in the first tube. Respectively pipette **36ul** Molecular Grade Water into next three tubes. Do three dilutions as the following figures:



To generate a standard curve on the real-time system, all four dilution standards should be used and defined as standard with specification of the corresponding concentrations.

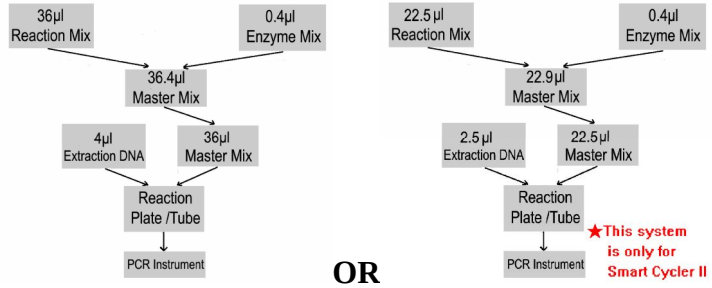
Attention:

A. Mix thoroughly before next transfer.

B. The positive control (1×10 copies/ml) contains high concentration of the target DNA. Therefore, be careful of dilution in order to avoid contamination.

9.3 PCR Protocol

The Master Mix volume for each reaction should be pipetted as follows:



1) The volumes of Reaction Mix and Enzyme Mix per reaction multiply with the number of samples, which includes the number of controls, standards, and sample prepared. Molecular Grade Water is used as the negative control. For reasons of unprecise pipetting, always add an extra virtual sample. Mix completely then spin down briefly in a centrifuge.

2) Pipet **36µl (22.5µl for Smart Cycler II)**Master Mix with micropipets of sterile filter tips to each *Real time* PCR reaction plate/tubes. Separately add **4µl (2.5µl for Smart Cycler II)**DNA sample, positive and negative controls to different reaction plate/tubes. Immediately close the plate/tubes to avoid contamination.

3) Spin down briefly in order to collect the Master Mix in the bottom of the reaction tubes.

4) Perform the following protocol in the instrument:

37°C for 2min	1cycle
94°C for 2min	1cycle
93°C for 15sec, 60°C for 1min (Fluorescence measured at 60°C)	40cycles

Selection of fluorescence channels	
FAM	Target Nucleic Acid

5) ⚠ If you use ABI Prism® system, please choose **"none"** as **passive reference** and **quencher.**

10. Threshold setting: just above the maximum level of molecular grade water.

11.Calibration for quantitative detection: Input each concentration of standard controls at the end of run, and a standard curve will be automatically formed.

12.Quality control: Negative control, positive control, and QS curve must be performed correctly, otherwise the sample results is invalid.

Control	Ct value in channel FAM
Molecular Grade Water	UNDET
Positive Control(qualitative assay)	≤35
QS（quantitative detection）	Correlation coefficient of QS curve≤－0.98

13. Data Analysis and Interpretation： The following sample results are possible:

	Ct value in Channel FAM	Result Analysis
1#	UNDET	Below the detection limit or negative
2#	≤38	Positive; and the software displays the quantitative value
3#	38～40	Re-test; If it is still 38~40, report as 1#

For further questions or problems,please contact our technical support at trade**@liferiver.com.cn**